Detection of oligonucleotides by PNA-peptide conjugates recognizing the biarsenical fluoresceine complex FlAsH-EDT_2

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Abstract

We report the application of the arsenical complex FlAsH-EDT_2 for the identification of oligonucleotide sequences. We designed PNA sequences conjugated to either a tetracysteine motif and to split tetracysteine sequences, that are recognized by FlAsH. The effect of conjugation of the PNA to the tetracysteine peptide and RNA hybridization on the fluorescence of the arsenical complex has been investigated. The reconstitution of the tetracysteine motif, starting from 15-mer PNAs conjugated to split tetracysteine sequences and hybridized to a complementary oligonucleotide was also explored.

Keywords: Peptide Nucleic Acids, FlAsH EDT_2, hybridization, duplex, MYCN, miR 17-5p

Introduction

Recent discoveries in the field of non-coding RNAs (ncRNAs) have greatly promoted the research of tools to detect, localize and monitor RNA molecules in living cells using fluorescent probes[1]. A number of strategies have been developed so far, based on the recognition of RNA sequences by fluorescent RNA binding proteins, RNA binding fluorescent tags or by complementary oligonucleotides bearing a fluorescent tag. When fluorescent probes are conjugated or incorporated into oligonucleotide sequences, hybrids can easily be detected; multiple fluorescent probes are usually employed to increase brightness. The background fluorescence of the proteins or of the probes limits the application of these systems. Fluorescent probes sensitive to the polarity of the environment are widely employed to reveal hybridization processes. These probes

are often covalently linked to the sugar portion of one nucleotide[2,3] and exhibit intense fluorescence upon hybridization to the targets, while very weak fluorescence when installed on single strands. Thiazole Orange (TO) is an example of light-up probe which has been demonstrated useful for the imaging of RNA/DNA counterparts in vitro and in vivo[4,5,6]. The use of metal complexes for the imaging of oligonucleotide is little explored. Radiolabeled PNA have been obtained and tested in vivo for radiopharmaceutical applications[7,8]. Luminescent Ru(I) complexes have been covalently linked at the N-terminus of peptide nucleic acid (PNA) oligomers; emission properties of the metal complexes do not change upon conjugation of the complex to the PNA oligomer and/or hybridization [9,10].

Peptides can also be employed as metal ligands. Metal complexes showing high specificity of binding toward specific peptide sequences have been reported [11,12,13,14]. The most successful example of metal complex exhibiting high affinity of binding toward a specific peptide sequence is represented by the 4',5'-bis(1,3,2-dithioarsolan-2-yl)fluorescein, namely FlAsH EDT_2[15]. FlAsH-EDT_2 is of great interest due to its brightness, selectivity toward the tetracysteine motif, ability to be taken up by cells[15]; it lights up upon complexation of the arsenic moiety to the tetracysteine motif and following displacement of the two 1,2 etanedithiol (EDT) molecules. Proteins bearing the tetracysteine motif can be identified in cellular systems with high sensitivity [16,17]. Interestingly, the tetracysteine motif can be split in two halves: when the two halves come close in the space, reconstitution of the tetracysteine motif occurs and this can be revealed by the fluorescence of the biarsenical complex. In this context, the FlAsH-EDT_2 may be used as a probe to detect either dimerization of two proteins and structural rearrangements of two protein domains, each linked to a half of the tetracysteine motif[18,19]. Analogous applications have been reported for resourcine-arsenical (ReAsH) complexes[20,21,22].

In this work, we explored the use of FlAsH-EDT_2 for the recognition of PNA/RNA and PNA/DNA hybrids. PNA oligomers are conjugated to a peptide sequence ("full" and "split") that is specifically recognized by FlAsH-EDT_2; when the split peptide is employed, the reconstitution of the tetracysteine motif is DNA templated. The high stability to degradation in vivo of PNAs, the high affinity and specificity of binding of PNA towards complementary DNA and RNA, together with the brightness and the cell permeability of the FlAsH complexes render this strategy very promising for the imaging of oligonucleotides[23,24,25]. We investigated the effect of conjugation of the PNA to the tetracysteine peptide on the fluorescence of the complex formed upon interaction of the FlAsH-EDT_2 with the tetracysteine peptide. We also monitored the effect of hybridization to RNA oligomers on the fluorescence of the PNA conjugated to the tetracysteine peptide and complexed with FlAsH . The PNA-tetracysteine conjugate was designed to target miR-17-5p while the PNAs conjugated to the split tetracysteine motifs target MYCN. Both targets are highly expressed in neuroblastoma (NB) cells and therefore useful as markers for NB[26,27]

Materials and Methods

Peptide and peptide-PNA conjugates synthesis. See Supplementary materials

Fluorescence Experiments.

The FlAsH-EDT_2 dye was prepared according to procedures described in the literature[25]. NMR and mass spectrometry results are consistent with those reported in the literature. Fluorescence experiments were performed according to published procedures [25].

Fluorescence experiments were carried out monitoring the fluorescence intensity at 530 nm upon excitation at 508 nm. Each experiment was performed in a buffer solution of 10mM aqueous

solution of 3-morpholinopropane-1-sulfonic acid (MOPS) neutralized with NaOH to pH 7.2 and degassed. The fluorescence of the complex formed by FlAsH with the peptide 4C was measured at 0.5 and 1 μM concentration. The concentration of PNA a17 4C, PNA a17 4C/RNA duplexes is 0.5μM, the concentration of the PNAs R8-PNA1 2C-C, N-2C-PNA2-R8 and of the corresponding duplexes with DNA is 1 μM. PNA/RNA (or DNA) duplexes were obtained mixing 10 μM of PNA and 10 μM of complementary RNA (or DNA) in MOPS buffer 100 mM, pH 7.2. The annealing reaction was performed by warming up to 90°C for 5 min, slowly cooling down to 4°C. Duplexes were kept at 4°C overnight before use. All experiments were repeated in duplicate. Experiments were carried out as follows:

to 2.5 mL of the MOPS buffer in a fluorescence cuvette, were added 25 μL of MESNa (1M in H_2O) followed by 2.5 μL of EDT (10 mM in dry DMSO) to give final concentrations of MESNa and EDT of 10 mM and 10 μM respectively and FlAsH-EDT_2 (from a 2mM stock solution prepared in dry DMSO). The final concentration of FlAsH-EDT_2 is, identical to that of the oligonucleotide added (0.5 or 1 μM). The fluorescence is measured every 5 min for 15-20 min or till the value stays constant.

Next single strand PNA (PNA a17 4C or R8-PNA1 2C-C + N-2C-PNA2-R8) or duplexes (PNA a17 4C/miR-17-5p or R8-PNA1 2C-C + N-2C-PNA2-R8/MYCN or N-2C-PNA2-R8 /MYCN) are added and the fluorescence measured every 15 min for the following 3 hours.

In a different experiment an equimolar amount of the miR-17-5p or the miR-17-5p mismatch 1, miR-17-5p mismatch 2 or miR-17-5p mismatch 3 was added to the solution of PNA-a17-4C+ FlAsH in MOPS EDT, prepared as described earlier.

Other control experiments were carried out measuring the fluorescence of solution obtained adding to the MESNa+EDT solution in MOPS the tetracysteine peptide 4C or miR17-5p (to be sure that the FlAsH does not interact with the RNA).

Results and discussion

Design and synthesis of PNA-peptide conjugates. The detection of DNA and RNA targets designated as markers of human diseases requires the development of probes highly sensitive. In this work, we investigated the ability of PNA conjugated to the peptide motif recognized by the arsenical complex FlAsH-EDT_2 and hybridized to complementary RNA and DNA sequences to light up upon formation of the metal complex.

We designed molecules for the imaging of miR-17-5p and of MYCN mRNA (Fig 1A).The molecules are composed of a PNA module, devoted to the recognition of the RNA target and a peptide motif which selectively recognizes and lights up the fluorophore FlAsH-EDT_2 upon displacement of the EDT molecules by the four cysteines motif[28]. For the recognition of miR-17-5p we designed a 23-mer PNA perfectly complementary to miR-17-5p and conjugated at the N terminus to the peptide PEMCCGPCCNLF (4C, “full” peptide sequence); the conjugate is named PNA a17 4C (Table 1). For the recognition of MYCN mRNA we envisioned the use of two 15-mer PNA oligomers, each conjugated to one of the halves of the tetracysteine motif (“split” peptide sequence) (Fig 1B) (Table 1). Conjugation of these PNAs to a polyarginine (R8) peptide is thought to be an effective strategy for enhancing cellular permeability for future in vitro and in vivo applications. The sequences of the “split” peptides are: PEMCC (2C-C) and CCNLF (N-2C). The two PNAs are complementary to contiguous region of the mRNA and, following hybridization to the mRNA, should enable the reconstitution of the tetracysteine peptide motif by allowing the cysteine residues to come close into space. The PNAs are named R8-PNA1 2C-C, when the polyarginine is

conjugated at the N-terminus and the split tetracysteine motif (2C-C) is at the C-terminus of the PNA, and N-2C-PNA2-R8 when the polyarginine is at the C-terminus of the PNA and the split tetracysteine peptide (N-2C) is at the N-terminus of the PNA. Each PNA (PNA1 and PNA2) is 15 bases long and together span the region of the N-myc mRNA that begins with the ATG start codon at position 1,650[29] This region of the MYCN sequence is reported as efficiently recognized by antisense complementary oligonucleotides[30,31,32]. Conjugates were obtained in all case by solid phase synthesis, using standard protocols [6,33,34].

Fluorescence experiments. Fluorescence experiments were performed according to procedures described in the literature[25]. We initially monitored the fluorescence emission intensity upon excitation at 508 nm of the FlAsH complexes formed with the peptide 4C and the single strand PNA-peptide conjugate PNA a17 4C. The fluorescence intensity of the complexes increases with the time and becomes stable after 180 minutes of incubation (Fig S1). For this reason we report the fluorescence intensity after 180 minutes of incubation of the arsenical complex with our target molecules. The FlAsH emission wavelength shifts from 528 nm in the complex with the tetracysteine peptide (4C) to 531 nm in the complexes with the PNA-peptide conjugate. We calculated the increase in the fluorescence intensity of the complexes with respect to the FlAsH-EDT_2 (i.e. fluorescence of the complex/fluorescence of FlAsH-EDT_2). A comparison between the fluorescence of the complex FlAsH-4C and FlAsH-PNA a17 4C revealed that the conjugation of the peptide to the PNA reduces the fluorescence intensity of the complex of about one third (Fig 2A). In fact, we observed a 300 fold increase in the fluorescence for the FlAsH-4C peptide complex and a 90 fold increase for the FlAsH-PNA a17 4C complex (Fig 2A). This result may be explained considering that conjugation of the PNA to the peptide changes the chemical environment of the complex, which in turn affects the fluorescence emission properties of the complex. Formation of

the hybrid upon addition of miR-17-5p to the complex formed by FlAsH and PNA a17 4C does not significantly affect the fluorescence of the complex (Fig 2A). Similarly, hybridization of the RNA oligomers miR-17-5p mismatch 1, miR-17-5p mismatch 2 and miR-17-5p mismatch 3 (Table 2), which are not perfectly complementary to PNA a17 4C to the complex formed by PNA a17 4C and FlAsH, does not change the fluorescence intensity emission of the complex. In fact the ratio between the fluorescence of the complexes before and after hybridization is comparable (Fig 2B), demonstrating that the complex is very stable and its properties are not affected by the stability of the duplexes. It is indeed expected that the insertion of mismatches reduces the thermal stability of the duplexes.

Next we investigated the complexation of FlAsH-EDT_2 to PNA/RNA duplexes obtained by annealing the PNA and RNA strands (Fig 2C). The fluorescence intensity of the complexes in this case is lower as compared to the fluorescence intensity of the complexes obtained hybridizing the RNA to the PNA a17 4C-FlAsH complex (compare Fig 2C and Fig 2A) and this is likely due to the steric and electronic effects imposed by the presence of the duplex which hampers the formation of the metal complex. Also in this case the formation of the complex is independent of the stability of the duplexes, within the experimental error. A 53 fold increase in the fluorescence intensity is observed when FlAsH is complexed to the duplex PNA a17 4C/miR-17-5p (calculated as fluorescence of the duplex/fluorescence of FlAsH EDT_2), (Fig 2C). When the PNA a17 4C is hybridized to RNA sequences not perfectly complementary, with one to three mismatches, the fluorescence signal observed upon complexation of the FlAsH-EDT_2 is comparable or slightly lower than that observed for the duplex PNA a17 4C/miR-17-5p. The ability to distinguish complementary matched and mismatched sequences is in fact low when the PNA oligomers are long; on the other side the use of long PNA sequences is dictated by the need to target unique RNA or DNA sequences, keeping in mind that the chance that a specific sequence is unique in the

genome is proportional to its length. We wish to highlight that the observed increase is higher than that observed with other probes such as TO attached as a tag at the end of a PNA oligomer[6] or perylene or pyrene incorporated into oligonucleotide sequences[3,35]. In the case of TO conjugated at the N-terminus of a PNA sequence we observed an increase of only 2.5-3 fold in the fluorescence intensity upon hybridization[6].

As controls we also measured fluorescence of DNA and RNA single strands mixed with FlAsH-EDT_2 and of duplex hybrids without FlAsH. As expected, single stranded PNA-peptide conjugates, single stranded DNA and RNA, duplexes formed between the PNA-peptide conjugates and either DNA and RNA oligomers are not fluorescent; also mixtures of DNA or RNA oligomers with FlAsH exhibit a very weak fluorescence whose intensity is identical to that shown by the FlAsH-EDT_2 complex in the same conditions. .

The biarsenical complex was employed to recognize longer oligonucleotide sequences. To this aim, we explored the complexation of the FlAsH-EDT_2 to the split tetracysteine motif. We used as a target a DNA oligomer having the same sequence as the MYCN mRNA and to allow the recognition of the longer sequence we used two PNA oligomers, each conjugated to a split tetracysteine motif. To grant selectivity we employed PNA sequences 15 bases long. The peptide sequences that have been conjugated to the PNAs are: PEMCC and CCNLF. The two PNAs, each conjugated to a split tetracysteine motif (namely R8-PNA1 2C-C and N-2C-PNA2-R8), were hybridized to complementary oligonucleotides of different length. The reconstitution of the tetracysteine sequence is templated and occurs upon hybridization (Fig 3). The hybrids differ for the distance, expressed in term of base pairs gap, between the two PNAs on the MYCN DNA. These experiments are aimed at finding the most favorable distance between the two PNAs which allows for the reconstitution of the tetracysteine motif and its subsequent reaction with FlAsH-EDT_2. We used

DNA sequences instead of RNA sequences, to reduce the cost of our experiments. We assume that the difference in the three-dimensional structure of both the PNA/DNA and PNA/RNA duplexes does not drastically affect the re-assembly of the tetracysteine ligand. As described earlier, the fluorescence intensity of the hybrids was compared to that of the FlAsH-EDT_2 complex in buffer. When PNAs R8-PNA1 2C-C and N-2C-PNA2-R8 hybridize the complementary oligonucleotide and there is no base pair gap between the PNAs, we observe a 38 fold increase in the fluorescence intensity upon complexation of FlAsH-EDT_2 (Fig 4A). When the base pair gap expands, the increase of the fluorescence intensity of the complex is lightly lower. The increase in the fluorescence intensity of the complex formed with the split tetracysteine motif is lower than that observed for the full tetracysteine motif. In this case, the length of the PNA probes is critical to determine the fluorescence of the complexes. In fact, in a recently published paper it is reported that hybridization of two 8-mer PNA sequences, equipped with a Pro-Ala-Gly-Cys-Cys peptide to a complementary DNA results in a 80 fold increase in the fluorescence intensity as compared to the fluorophore[36]. Also, the use of two 8-mer PNAs enables the recognition of one base mismatch on the complementary oligonucleotide.

As a control, we monitored the fluorescence of the mixture of PNAs R8-PNA1 2C-C and N-2C-PNA2-R8 in the presence of FlAsH-EDT_2 and we found that the increase of the fluorescence intensity is comparable to that observed for the peptide 4C incubated with the biarsenical complex. We also measured the fluorescence of the duplex formed by N-2C-PNA2-R8 and MYCN and we observed a weak increase in the fluorescence intensity (Fig 4B). Complexation of FlAsH-EDT_2 occurs only with the reconstituted tetracysteine motif. Importantly, the use of two PNA sequences results in an increase in the specificity of the systems, as a larger number of bases need to be recognized by the PNAs.

The results of our studies demonstrate that the FlAsH-EDT_2 lights up when the tetracysteine peptide is conjugated to a PNA sequence as single strand or hybridized to its complementary oligonucleotide.. Results obtained here encourage the application of the tetracysteine motif for the detection of oligonucleotide sequences *in vivo*.

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References

[1] B.A. Armitage, Imaging of RNA in live cells, Current opinion in chemical biology 15 (2011) 806-812.

[2] M.E. Ostergaard, P. Cheguru, M.R. Papasani, R.A. Hill, P.J. Hrdlicka, Glowing locked nucleic acids: brightly fluorescent probes for detection of nucleic acids in cells, Journal of the American Chemical Society 132 (2010) 14221-14228.

[3] I.V. Astakhova, V.A. Korshun, K. Jahn, J. Kjems, J. Wengel, Perylene attached to 2'-amino-LNA: synthesis, incorporation into oligonucleotides, and remarkable fluorescence properties in vitro and in cell culture, Bioconjugate chemistry 19 (2008) 1995-2007.

[4] A. Okamoto, ECHO probes: a concept of fluorescence control for practical nucleic acid sensing, Chemical Society reviews 40 (2011) 5815-5828.

[5] S. Kummer, A. Knoll, E. Socher, L. Bethge, A. Herrmann, O. Seitz, Fluorescence imaging of influenza H1N1 mRNA in living infected cells using single-chromophore FIT-PNA, Angewandte Chemie 50 (2011) 1931-1934.

[6] C. Avitabile, M. Saviano, L. D'Andrea, N. Bianchi, E. Fabbri, E. Brognara, R. Gambari, A. Romanelli, Targeting pre-miRNA by peptide nucleic acids: a new strategy to interfere in the miRNA maturation, Artificial DNA, PNA & XNA 3 (2012) 88-96.

[7] G. Mardirossian, K. Lei, M. Rusckowski, F. Chang, T. Qu, M. Egholm, D.J. Hnatowich, In vivo hybridization of technetium-99m-labeled peptide nucleic acid (PNA), Journal of nuclear medicine : official publication, Society of Nuclear Medicine 38 (1997) 907-913.

[8] G. Gasser, K. Jager, M. Zenker, R. Bergmann, J. Steinbach, H. Stephan, N. Metzler-Nolte, Preparation, 99mTc-labeling and biodistribution studies of a PNA oligomer containing a new ligand derivative of 2,2'-dipicolylamine, Journal of inorganic biochemistry 104 (2010) 1133-1140.

[9] E. Ferri, D. Donghi, M. Panigati, G. Prencipe, L. D'Alfonso, I. Zanoni, C. Baldoli, S. Maiorana, G. D'Alfonso, E. Licandro, Luminescent conjugates between dinuclear rhenium(I) complexes and peptide nucleic acids (PNA) for cell imaging and DNA targeting, Chemical communications 46 (2010) 6255-6257.

[10] G. Gasser, A. Pinto, S. Neumann, A.M. Sosniak, M. Seitz, K. Merz, R. Heumann, N. Metzler-Nolte, Synthesis, characterisation and bioimaging of a fluorescent rhenium-containing PNA bioconjugate, Dalton transactions 41 (2012) 2304-2313.

[11] A. Ojida, K. Honda, D. Shinmi, S. Kiyonaka, Y. Mori, I. Hamachi, Oligo-Asp tag/Zn(II) complex probe as a new pair for labeling and fluorescence imaging of proteins, Journal of the American Chemical Society 128 (2006) 10452-10459.

[12] K. Honda, S.H. Fujishima, A. Ojida, I. Hamachi, Pyrene excimer-based dual-emission detection of a oligoaspartate tag-fused protein by using a Zn(II)-DpaTyr probe, Chembiochem : a European journal of chemical biology 8 (2007) 1370-1372.

[13] A.N. Kapanidis, Y.W. Ebright, R.H. Ebright, Site-specific incorporation of fluorescent probes into protein: hexahistidine-tag-mediated fluorescent labeling with (Ni(2+):nitrilotriacetic Acid (n)-fluorochrome conjugates, Journal of the American Chemical Society 123 (2001) 12123-12125.

[14] C.R. Goldsmith, J. Jaworski, M. Sheng, S.J. Lippard, Selective labeling of extracellular proteins containing polyhistidine sequences by a fluorescein-nitrilotriacetic acid conjugate, Journal of the American Chemical Society 128 (2006) 418-419.

[15] B.A. Griffin, S.R. Adams, R.Y. Tsien, Specific covalent labeling of recombinant protein molecules inside live cells, Science 281 (1998) 269-272.

[16] Z. Ignatova, L.M. Gierasch, Monitoring protein stability and aggregation in vivo by real-time fluorescent labeling, Proceedings of the National Academy of Sciences of the United States of America 101 (2004) 523-528.

[17] Z. Ignatova, B. Krishnan, J.P. Bombardier, A.M. Marcelino, J. Hong, L.M. Gierasch, From the test tube to the cell: exploring the folding and aggregation of a beta-clam protein, Biopolymers 88 (2007) 157-163.
[18] B. Krishnan, L.M. Gierasch, Cross-strand split tetra-Cys motifs as structure sensors in a beta-sheet protein, Chemistry & biology 15 (2008) 1104-1115.
[19] N.W. Luedtke, R.J. Dexter, D.B. Fried, A. Schepartz, Surveying polypeptide and protein domain conformation and association with FlAsH and ReAsH, Nature chemical biology 3 (2007) 779-784.
[20] R.A. Scheck, A. Schepartz, Surveying protein structure and function using bis-arsenical small molecules, Accounts of chemical research 44 (2011) 654-665.
[21] Y. Ma, Z. He, T. Tan, W. Li, Z. Zhang, S. Song, X. Zhang, Q. Hu, P. Zhou, Y. Wu, X.E. Zhang, Z. Cui, Real-Time Imaging of Single HIV-1 Disassembly with Multicolor Viral Particles, ACS nano 10 (2016) 6273-6282.
[22] R.J. Dexter, A. Schepartz, Direct visualization of protein association in living cells with complex-edited electron microscopy, Angewandte Chemie 49 (2010) 7952-7954.
[23] M. Eriksson, P.E. Nielsen, PNA nucleic acid complexes. Structure, stability and dynamics, Quarterly Reviews of Biophysics 29 (1996) 369-394.
[24] V.V. Demidov, V.N. Potaman, M.D. Frankkamenetskii, M. Egholm, O. Buchard, S.H. Sonnichsen, P.E. Nielsen, Stability of Peptide Nucleic-Acids in Human Serum and Cellular-Extracts, Biochemical Pharmacology 48 (1994) 1310-1313.
[25] S.R. Adams, R.Y. Tsien, Preparation of the membrane-permeant biarsenicals FlAsH-EDT2 and ReAsH-EDT2 for fluorescent labeling of tetracysteine-tagged proteins, Nature protocols 3 (2008) 1527-1534.
[26] L.M. Wagner, M.K. Danks, New therapeutic targets for the treatment of high-risk neuroblastoma, Journal of cellular biochemistry 107 (2009) 46-57.
[27] Y. Chen, R.L. Stallings, Differential patterns of microRNA expression in neuroblastoma are correlated with prognosis, differentiation, and apoptosis, Cancer research 67 (2007) 976-983.
[28] B.R. Martin, B.N. Giepmans, S.R. Adams, R.Y. Tsien, Mammalian cell-based optimization of the biarsenical-binding tetracysteine motif for improved fluorescence and affinity, Nature biotechnology 23 (2005) 1308-1314.
[29] L.W. Stanton, M. Schwab, J.M. Bishop, Nucleotide sequence of the human N-myc gene, Proceedings of the National Academy of Sciences of the United States of America 83 (1986) 1772-1776.
[30] N. Watanabe, H. Sawai, I. Ogihara-Umeda, S. Tanada, E.E. Kim, Y. Yonekura, Y. Sasaki, Molecular therapy of human neuroblastoma cells using Auger electrons of 111In-labeled N-myc antisense oligonucleotides, Journal of nuclear medicine : official publication, Society of Nuclear Medicine 47 (2006) 1670-1677.
[31] A. Negroni, S. Scarpa, A. Romeo, S. Ferrari, A. Modesti, G. Raschella, Decrease of proliferation rate and induction of differentiation by a MYCN antisense DNA oligomer in a human neuroblastoma cell line, Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research 2 (1991) 511-518.
[32] C.A. Burkhart, A.J. Cheng, J. Madafiglio, M. Kavallaris, M. Mili, G.M. Marshall, W.A. Weiss, L.M. Khachigian, M.D. Norris, M. Haber, Effects of MYCN antisense oligonucleotide administration on tumorigenesis in a murine model of neuroblastoma, Journal of the National Cancer Institute 95 (2003) 1394-1403.
[33] C. Avitabile, L. Moggio, L.D. D'Andrea, C. Pedone, A. Romanelli, Development of an efficient and low-cost protocol for the manual PNA synthesis by Fmoc chemistry, Tetrahedron Letters 51 (2010) 3716-3718.
[34] C. Avitabile, F. Netti, G. Orefice, M. Palmieri, N. Nocerino, G. Malgieri, L.D. D'Andrea, R. Capparelli, R. Fattorusso, A. Romanelli, Design, structural and functional characterization of a Temporin-1b analog active against Gram-negative bacteria, Biochimica et biophysica acta 1830 (2013) 3767-3775.
[35] M.E. Ostergaard, D.C. Guenther, P. Kumar, B. Baral, L. Deobald, A.J. Paszczynski, P.K. Sharma, P.J. Hrdlicka, Pyrene-functionalized triazole-linked 2'-deoxyuridines-probes for discrimination of single nucleotide polymorphisms (SNPs), Chemical communications 46 (2010) 4929-4931.

[36] G.M. Fang, O. Seitz, Bivalent Display of Dicysteine on Peptide Nucleic Acids for Homogenous DNA/RNA Detection through in Situ Fluorescence Labelling, Chembiochem : a European journal of chemical biology 18 (2017) 189-194.

Figure 1. Schematic illustration of the PNA-peptide conjugates employed. A: the tetracysteine motif (4C) conjugated to the PNA for the recognition of the target miR-17-5p; B: the split tetracysteine motif peptides (2C-C and N-2C) are conjugated to PNA oligomers complementary to the target MYCN mRNA.

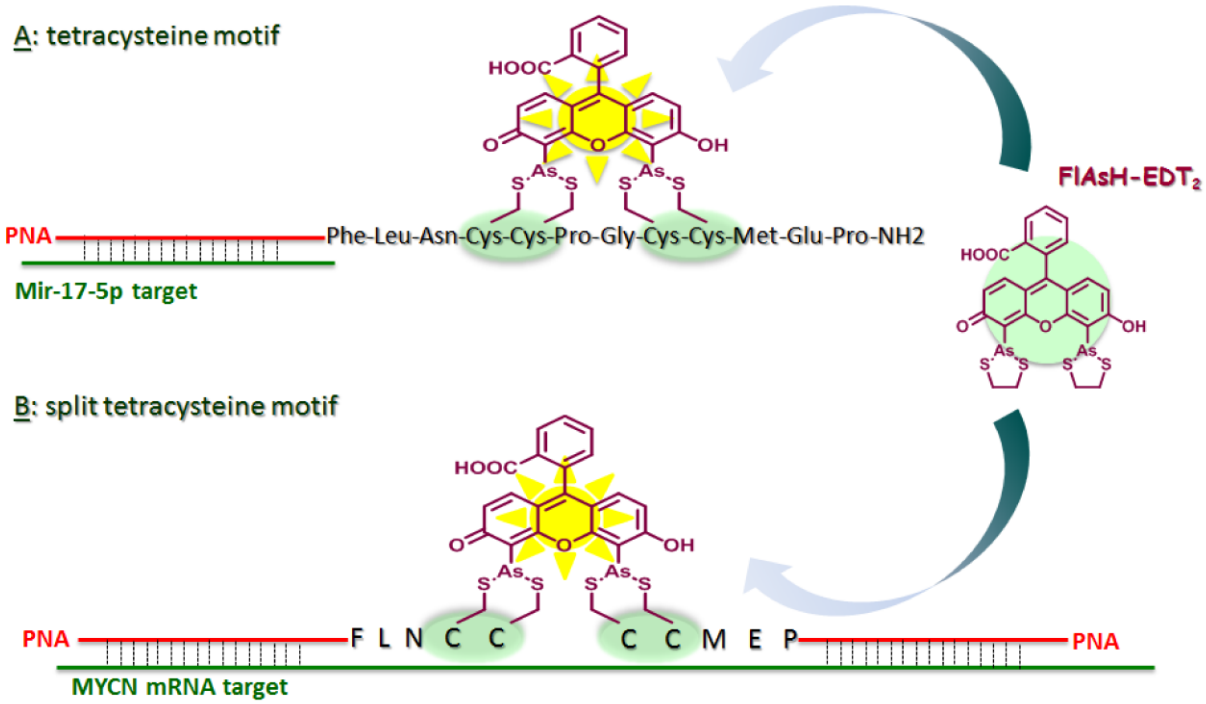


Figure 2. 2A: Effect of conjugation and hybridization on the fluorescence emission of the complexes formed by the FlAsH. Fluorescence of the complex peptide 4C +FlAsH (1) is compared to the fluorescence of the complexes PNA a17 4C+FlAsH (2) and PNA a17 4C+FlAsH+miR-17-5p (3). miR-17-5p is added to the complex of PNA-a17-4C and FlAsH. The fluorescence increase is measured as fluorescence of the complex/fluorescence of FlAsH EDT_2. The concentration of the peptide 4C, the PNA-peptide conjugate PNA a17 4C, miR-17-5p and FlAsH is 0.5μM. **2B:** Effect of the hybridization of the PNA a17 4C-FlAsH complex with the complementary miR-17-5p (1) and with mismatched sequences 1,2 and 3 (bar numbers 2,3,and 4). Sequences of the RNA are listed in table 2.The fluorescence is expressed as fluorescence of the FlAsH-duplex complex/fluorescence of the PNA a17 4C-FlAsH complex. The concentration of the PNA oligomers, the RNA and the fluorescent probe is 0.5μM. **2C**: Complexation of the FlAsH with PNA/RNA duplexes. In this experiment the duplexes were first annealed and then the FlAsH-EDT_2 complex was added. 1: duplex PNA a17 4C/ miR-17-5p; 2: duplex PNA a17 4C/ miR-17-5p mismatch 1; 3: duplex PNA a17 4C/ miR-17-5p mismatch 2; 4: duplex PNA a17 4C/ miR-17-5p mismatch 3. The fluorescence increase is expressed as fluorescence of the FlAsH-duplex complex/fluorescence of FlAsH-EDT_2. The concentration of the duplexes and of the FlAsH is 0.5μM.

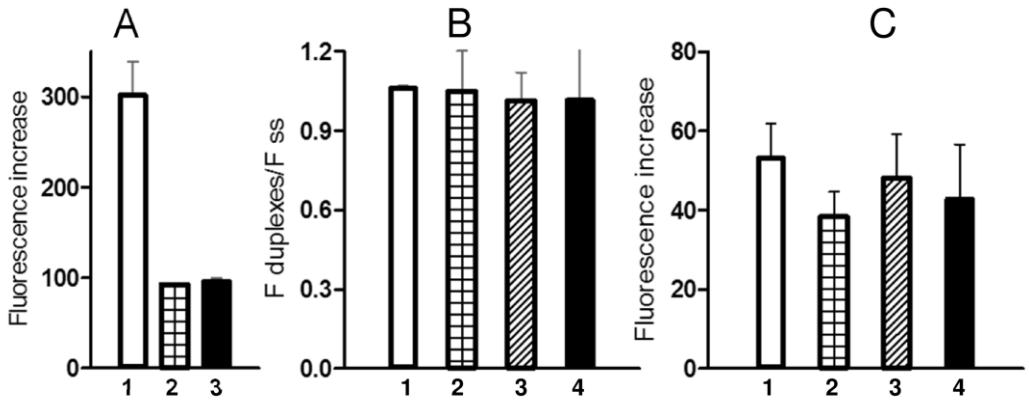


Figure 3. Schematic representation of the hybrids formed by the PNAs conjugated to the split tetracysteine motif and the complementary DNA sequence. Hybrids differ for the distance between the PNA sequences, which affects the distance between the split tetracysteine motifs**.**

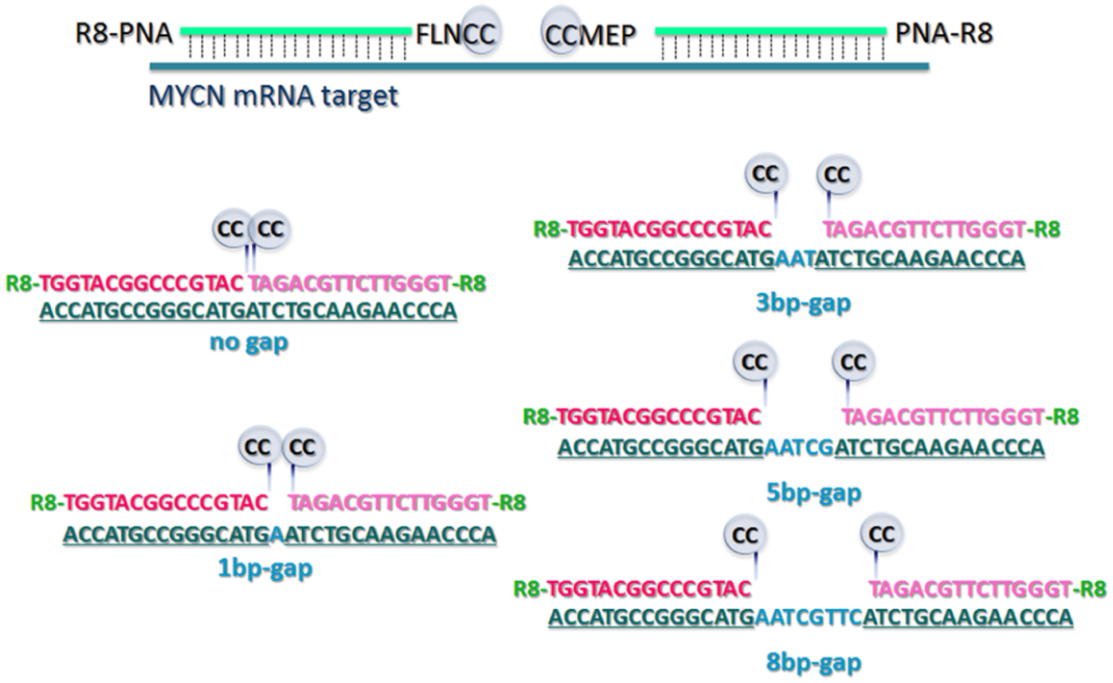
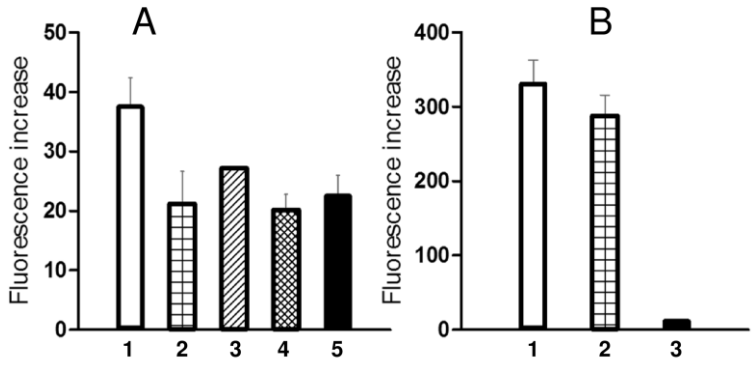


Figure 4. 4A: Fluorescence intensity increase of the duplexes formed by the PNAs conjugated to the split tetracysteine peptides and oligonucleotides of different length. PNAs are annealed to a complementary oligonucleotide with consecutive bases (no gap) (1) or to sequences separated by 1 (2), 3 (3), 5 (4) to 8 (5) base pair gaps, then the FlASH-EDT_2 is added. The increase in calculated as fluorescence of the FlAsH-duplex complex/fluorescence of the FlAsH-EDT_2. The scheme of the hybrids is reported in figure 3. The concentration of the duplexes and of the probe is 1μM. **4B**: Comparison of the fluorescence intensity of the complex formed by the FlAsH with the peptide 4C (1), an equimolar mixture of the PNAs R8-PNA1 2C-C+ N-2C-PNA2-R8 (2) , and the hybrid R8-PNA1 2C-C+ MYCN no gap (3) . The concentration of the PNAs, duplex and peptide is 1μM. The fluorescence reported for the complex 4C-FlAsH was measured after 120 minutes of incubation of FlAsH EDT_2 with the peptide, as at 180 minutes incubation was over-range**.**



Supporting Information content: Materials and general procedures, Peptide and peptide-PNA conjugates synthesis, Figure S1.

Figure S1